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Developmental and Comparative Immunology 28 (2004) 635–645
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Gene expression profiling of bovine macrophages in response to *Escherichia coli* O157:H7 lipopolysaccharide

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Received 27 June 2003; revised 3 October 2003; accepted 6 October 2003

Abstract

The aim of this study was to identify changes in bovine macrophage gene expression in response to treatment with *Escherichia coli* 0157:H7 lipopolysaccharide (LPS), utilizing a human gene microarray. Bovine cDNA from control and LPS-treated primary macrophages hybridized to greater than 5644 (79.8%) of the non-control gene targets on a commercially available microarray containing greater than 7075 targets (Incyte Genomics, St. Louis, MO). Of these target sequences, 44 were differentially expressed upon exposure to LPS, including 18 genes not previously reported to exist in cattle. These included a pentaxin-related gene, CASP8, TNF-induced genes, interferon-induced genes, and inhibitors of apoptosis. Using the human microarray, cDNA from bovine LPS-treated and control macrophages consistently hybridized to targets known to be expressed constitutively by macrophages, as expected given the predicted cDNA sequence homology. That this human system was accurately estimating levels of bovine transcripts was further verified by real-time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) using bovine-specific primers. This first report of bovine–human cross-species expression profiling by microarray hybridization demonstrates the utility of this technique in bovine gene expression and discovery.
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Keywords: *E. coli* O157:H7; Macrophage; Microarray; Cross-species hybridization; Bovine; Real-time quantitative PCR; LPS; Gene expression

1. Introduction

Identifying genes that affect susceptibility to mucosal pathogens of livestock will facilitate breeding of disease resistant livestock and also provide novel insights into the pathogenesis of infectious disease. *Escherichia coli* O157:H7 is an important human pathogen that can be found in the intestines of cattle, and shed in their feces. We have previously

Abbreviations: DEPC, diethyl pyrocarbonate; MARC, U.S. Meat Animal Research Center; NOS2, nitric oxide synthase 2; RAP-PCR, random arbitrarily-primed polymerase chain reaction; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcriptase polymerase chain reaction; RTQ-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; TE, Tris-EDTA.

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0145-305X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.dci.2003.10.002

used techniques such as RNA fingerprinting by random arbitrarily-primed polymerase chain reaction (RAP-PCR) to identify changes in bovine transcript abundance in response to *E. coli* O157:H7 lipopolysaccharide (LPS) [1], as well as semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to measure differences in the expression of bovine cytokines [2–5]. These techniques are limited in that they may not be sensitive enough to identify subtle changes in gene expression (in the case of RAP-PCR) or can only measure expression of a single gene at one time (RT-PCR). However, the advent of DNA microarray technology has provided a sensitive tool to concurrently evaluate expression of thousands of target sequences [6]. This technology is extremely useful in understanding the interrelationship of gene expression involved in the immune system and non-immune pathways. Using traditional methods, similar studies would require the daunting task of separately analyzing each gene in question.

Many growth factors, cytokines, and antibodies are functionally reactive across species and nucleotide sequence homology among animal species can be considerable in many genes [7]. Therefore, it is common for human gene sequences to be used to design probes for Northern and Southern analyses or primers for PCR analyses of animal genes [8–11]. Based on our past experience utilizing human DNA sequence to study bovine gene expression [1,2,12], we hypothesized that the homology between human and bovine genes was high enough to enable us to utilize commercially available microarrays manufactured using human gene targets to measure gene expression in bovine macrophages treated with *E. coli* O157:H7 LPS.

2. Materials and methods

2.1. Animals

Approximately, 480 ml of blood were obtained from each of 44 healthy beef cattle by jugular venipuncture into 60 cc heparinized syringes. Animals were of both sexes and housed at the U.S. Meat Animal Research Center (MARC) feedlot in accordance with USDA animal care guidelines.

2.2. Isolation of peripheral blood monocytes

PBMC were isolated from heparinized blood by density gradient centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden), as per the manufacturer's instructions, and erythrocytes removed using red blood cell lysing buffer (Sigma Chemical Co., St. Louis, MO). After washing with PBS, PBMC were resuspended in 40 ml of FBS-free Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine and dispensed into four to eight 75-cm^2 tissue culture flasks. Monocytes were isolated by adherence after culturing the cells for 1 h at 37 °C, 5% CO_2 followed by washing with PBS to remove non-adherent cells. Following adherence, monocyte-derived macrophage cultures were maintained overnight at 37 °C, 5% CO_2 in RPMI 1640 medium with L-glutamine, penicillin–streptomycin, and 5% FBS.

2.3. Purification of LPS

Purified LPS from *E. coli* O157:H7 was prepared as previously described [13]. Activity of the LPS was determined using the E-Toxate limulus amoebocyte kit (Sigma Chemical Co.), following the manufacturer's instructions. This assay is sensitive to 0.05–0.1 endotoxin units per ml. All other reagents used in the study were determined to be endotoxin-free by the same method.

2.4. Treatment with LPS

Control cultures were washed with PBS to remove non-adherent cells and 10 ml of culture medium was replaced. LPS-treated monocytes were maintained as described for the controls, however, 10 μg/ml *E. coli* O157:H7 LPS was added to the medium. Cells were cultured for an additional 3 h, washed twice with 10 ml PBS to remove exogenous protein from the cell cultures, lysed with 1 ml guanidine isothiocyanate, and frozen within the culture flasks at −80 °C until RNA isolation.

2.5. Isolation of RNA

Total RNA was extracted using phenol-chloroform as described [14]. Pellets were resuspended in 40 μl

of diethyl pyrocarbonate (DEPC)-treated water, and nucleic acid concentration and purity determined by absorbance at 260 nm. LPS-treated and non-treated samples were combined into respective pools and mRNA isolated using OligoTex mRNA isolation columns (Qiagen, Chatsworth, CA), as per the manufacturer's instructions. Samples were precipitated, resuspended in either DEPC water or Tris–EDTA (TE) at a concentration of 50 ng/μl and submitted to Incyte Genomics (St. Louis, MO) for hybridization to a human UniGEM V 1.0 microarray.

2.6. Microarray hybridization and analysis

Microarray hybridization and fluorescent signal measurements were performed by Incyte Genomics as previously described [15]. Briefly, human UniGEM V 1.0 microarrays were generated by arraying PCR amplified sequences onto glass slides. The LPS-treated and non-treated bovine macrophage mRNA pools were reverse-transcribed with 5'-fluorescently-labeled random 9-mers to generate LPS-treated and non-treated probe solutions (LPS and control, respectively). In order to differentiate between the probe solutions, each was labeled with a different fluorochrome (LPS and control labeled with Cy3 and Cy5, respectively). Probe solutions were simultaneously applied to the microarray for hybridization to target sequences. Hybridization stringency was proprietary, but identical to that used for human cDNA.

The microarray was scanned simultaneously with two independent lasers to detect fluorescence from the two fluorochromes. The signals were digitized in order to provide electronic images for visual representation. Incyte GEMtools 2.4 software (Incyte Pharmaceuticals, Inc., Palo Alto, CA) was used for image analysis. LPS:control ratios were calculated for all elements and were used to generate a "balance coefficient" that was applied in order to balance or normalize the signals.

2.7. RTQ-PCR verification of hybridization specificity

Bovine-specific primers were designed using Primer Express version 1.0 (Applied Biosystems, Foster City, CA) software for all genes that were significantly altered in expression as measured by microarray analysis, to verify that hybridization to the microarray targets was specific and repeatable (Table 1). Bovine-specific sequences were identified in the MARC bovine expressed sequence tag libraries [16] or in GenBank, by using the human sequence contained in GenBank to search for similar bovine sequences using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). RNA samples were treated with DNA-free™ (Ambion, Austin, TX) to ensure that all DNA had been removed. Assays were assembled using the Ambion MessageSensorRT kit (Austin, TX) as per the manufacturer's instructions. Briefly, a master mix was prepared for a one step RT-PCR reaction containing 5.8 μl nuclease-free water, 2.5 μl 10× RT-PCR buffer + SYBR Green I (stock of 10,000× diluted 100-fold in nuclease-free water; Molecular Probes, Inc., Eugene, OR), 2.5 μl 10× glycerol, 4 μl dNTP mix (2.5 mM each), 1 μl RNase inhibitor (10 U/μl), 0.5 μl 50× ROX internal reference (250 μM; Stratagene, La Jolla, CA), 0.2 μl Taq polymerase (5 U/μl), 2.5 μl 10× gene-specific RT-PCR primer mix (1 μM each of the gene-specific forward and reverse primers), 1 μl reverse transcriptase (1 U/μl), and 5 μl RNA (50 ng). Reactions for each gene were prepared in duplicate for both LPS-treated and control RNA, and minus-RT controls and minus-template controls were performed simultaneously. The RTQ-PCR reaction was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in either 8-well strips or 96-well plates as follows: Stage 1, 42°C for 15 min; Stage 2, 95°C for 5 min; Stage 3, 95°C for 15 s, 60°C for 30 s, and 76°C for 50 s. Stage 3 was repeated 39 times followed by a three-stage melting-curve program to ensure amplification of a single product: Stage 1, 95°C for 15 s; Stage 2, 60°C for 20 s; and Stage 3, a 20-min ramp to 95°C for 15 s with data collected every 7 s. All PCR reactions were normalized to bovine ubiquitin-C, and differential expression was determined using the comparative C_T method (User Bulletin #2 ABI Prism 7700 Sequence Detection System).

2.8. Determination of homology between human and bovine sequences

In order to estimate the homology between the human and bovine gene sequences utilized in this

Table 1
Oligonucleotides[a] for RTQ-PCR analysis of bovine gene expression

Gene symbol	Human accession number	Bovine accession number	% Homology between human and bovine sequences[b]	Forward primer	Reverse primer	Amplicon size (bp)	Differential expression[cd]	BDE[e]
PTX3	NM_002852	BE664288	87 (468/534)	TCTTTATTTATCTTGGCAAAAT ACTGAGTAA	AAGCACCATGGCATAAAATCTAGTAA	98	86.8	17.8
TNFSF2	M10988	BM255342	88 (338/380)	CCGGTGGTGGGACTCGTAT	GCTGGTTGTCTTACAGCTTCACA	65	3.6	4.3
TNFAIP6	M31165	BE666586	91 (491/534)	GCAGTTAGAGGCAGCCAGAAA	TCTGCCCTTGGCCATCC	67	1.7	4.2
CFLAR	AF015450	BF601020	89 (215/239)	AGTCAGCTCAAGGAGCGGAA	AGGATCTTTAACACTCAAGTTTGGC	77	3.6	3.9
BF	NM_001710	BE845981	88 (469/530)	GCTCTGCCAATCGCACCT	CATCACAGATGGCCGTTTCC	63	−2.5	3.7
BIRC2	U37547	BF654068	80 (399/497)	CCGGAAGAATAGAATGGCACTT	TCACCCTGGCAGTTAGGAGACT	80	1.1	3.2
BIRC3	U37546	BF654068	88 (440/497)	CCGGAAGAATAGAATGGCACTT	TCACCCTGGCAGTTAGGAGACT	80	1.1	3.0
NOS2A	U31511	AW654110	91 (504/552)	CCACCAACAACGGCAACA	TCCCATCGCTCCGCTG	60	1.5	3.0
MX1	NM_002462	NM_173940	81 (1237/1514)	CGCATCTCCGGCCACA	CTGCTCGCCATACGTCCG	63	1.7	2.9
SERPIND1	M58600	CB434316	83 (230/277)	ATACGAGACGCACTGCAGGA	TAGAGGCAGTTGAGGAGCAGC	62	2.1	2.9
IL1RA	X52015	BE484390	82 (196/239)	GCTCTTCCTGTTCCGTTCAGA	TGCATCTCGCAGCGTCTCT	63	1.2	2.7
ARG2	U75667	BF652843	89 (412/460)	GCCCTGGACCTCGTTGAA	TGTAGCCTTGGCCTCCTCCT	63	2.4	2.5
SCYA20	D86955	AW660024	79 (290/366)	TCTTGTGGGCTTCACACAGC	TTTCCTGGTGTAAAAGACAACTGC	73	18.7	2.5
NFKBIA	AI906005	BI540594	94 (446/473)	CCATGAAGAGAAGGCGCTG	AGGAAGGCCAGGTCTCCCT	63	1.8	2.4
CSF3	M17706	BM256261	89 (288/321)	CTTGGCCCTGCCCGA	TTCCTCACTTGCTCTAAGCACTTG	62	1.5	2.3
INDO	NM_002164	BE485813	80 (378/472)	CACAGCGCCTGGCACAC	CGCCTTGACCCCACACAT	63	1.5	2.3
TIMP1	AI952703	BI541224	85 (374/435)	GCACATCACCACCTGCAGTT	CCCGGCGCTGAGCA	62	4.1	2.3
IL1A	M28983	NM_174092	82 (682/826)	AGTTGCCCATCCAAAGTTGTTT	GCGGCCCACTTGCCA	68	1.2	2.3
NFKB2	S76638	BF652508	90 (432/476)	TATACCCAGTCCACCTGGCAG	TCTCCACCAGCAGATCGAGG	63	1.8	2.2
cig5	AF026941	CB452670	85 (251/294)	GAAGCGGAACGGTTCGTG	ACATCCTTGTGGCGATCCA	65	1.7	2.2
ID2	AI191485	BG692795	89 (334/372)	CAGTGAGGTCCGTGAGGAAAA	TTGCTCCGGGAGATGCC	61	1.7	2.2
IFIT1	M24594	No SEQUENCE						2.2
SLC2A3	AW163231	BE899741	81 (424/521)	TTGGAAGAGCGGTCAGAACC	ACAGACAAGGACCACAGGGATG	65	2.5	2.2
TNFAIP3	AI474055	CB446530	80 (78/97)	TGCTAAGCTGGCTGCAAGG	TTGCTGTGTTGATGCTGCG	59	1.3	2.2
MT1L	F26407	BE667089	84 (202/238)	ACCCTCGCCATCCTTTGC	ATCCATGGCGAGCTGAACTG	63	3.8	2.1
CASP4	U25804	AV618261	82 (474/574)	CATATGCCTCCCAGGAATGG	GCCAAGACCCTCAAGCAGC	69	1.2	2.1
IFITM1	BF665518	NM_174551	82 (317/386)	CTTGACGACCACGGTGATCA	GACCAGACGATGTGGTCGG	63	1.8	2.1
ISG15	AI739106	NM_174366	76 (362/476)	ATCAATGTGCCTGCTTTCCAG	TCCTGCAGCACCTCCCTG	62	−1.3	2.1
MX2	M33883	NM_173941	81 (1513/1861)	CGGAATCATCACCCGGTG	CCCGGTCCATTCACACTCC	64	3.8	2.1
PTGS2	D28235	AF031698	87 (453/517)	GCACAAATCTGATGTTTGCATTC	GGTCCTCGTTCAAAATCTGTCTTG	76	6.0	2.1
TOP1	M60706	BE808376	96 (242/251)	TGTGAAGACATTTTTTGCTATAA TCATTAG	GGAGAGATGTGGGAAATGGACT	86	2.3	2.1
ALAS1	AA707391	AV665498	83 (272/324)	CTTTGCGGAAGACACTGCTG	TGGGAGCAAATGCCCTTTC	65	1.6	2.0
BCL2A1	NM_004049	BI539243	81 (468/571)	GCAGATACAGCAACCTGGATCC	CTGGACAGAGGAAGCCACATC	73	1.7	2.0
IFIT4	AF083470	BF776617	89 (252/281)	GCACTTTGGGAGGCCGA	TGTTAGCCAGGATGGTATCGATC	64	−10.0	2.0
CD36	NM_000072	AW486990	*	GCAAGAATGGCGCACCTATT	CAATGGCAGAGACAAACTTTTCA	75	−2.0	−3.7
FABP5	AA931982	BF430188	88 (394/447)	GGCTCTGCGAAAAGTGGGT	TGCTGAGGTTTTTGCCATCA	71	−10.0	−3.5
LGALS3	AB006780	BM258297	88 (345/389)	TTGCTTTAGATTTCAAGAGAGGGAA	TGACTCTCCTAGTTGTCCTCAATGA	79	−5.0	−2.5
TGFBI	M77349	BI847961	90 (501/551)	TCCTGGCCACCAACGG	GCGTCTTGGCTGAGTCAGGT	66	−2.5	−2.5
PGD	U30255	AW487342	86 (416/483)	TTGGCTGGACCCTCAACTATG	TGATGATGCAGCCCCCC	60	−3.3	−2.4
TXNIP	S73591	BE589784	90 (456/504)	AGCGTCCCTGGCTCCAA	GCCTGACCTGCTGCCAATA	63	−1.3	−2.3
LGMN	NM_005606	BE590231	84 (438/519)	TGGAGGACCCTGAGGACG	TACCAGCCGTTTGATCCTGC	64	−5.0	−2.1
VIM	X56134	AV594041	93 (532/571)	CGAGGTGGAGCGCGAC	TCCTGCAACTTCTCCCGG	60	−5.0	−2.0
NR1H3	NM_005693	AV610080	90 (616/677)	CGGAGGCTCACCAGTTTCA	CCATCCACCACCCCCAT	64	−5.0	−2.0

THBS1	NM_003246	AV616902	93 (497/533)	TCCCTGTCATCTGGAATCTTATCA	CAAGGATGGAATCGGCGA	66	− 2.0	− 2.0	
Ubiquitin C	AA578839	BE668033	*	TCCCTACCTGCATCATGTGC	GGAATTTGGGCCAGTGCTC	71	House-keeping gene	1.1	

[a] Sequences listed 5′ to 3′.

[b] Percent homology between human and bovine sequences was determined using bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) with filters "off". Stars indicate homology between specific sequences was not significant. The bovine sequences used were selected by homology to other human sequences specific for these genes.

[c] Values in gray boxes are not in agreement with microarray results.

[d] Differential expression measured using the formula $2^{-\Delta\Delta Ct}$ where Ct of the housekeeping gene is subtracted from the Ct of the specific gene from LPS-treated samples (ΔCts), subsequently, the Ct of the housekeeping gene is subtracted from the Ct of the specific gene from untreated samples (ΔCtc). Finally, ΔCts − ΔCtc = ΔΔCt. In order to report the reduced differential expression of genes as measured by RTQ-PCR in the same format as the microarray results, these values were further transformed using the formula $-1/(2^{-\Delta\Delta Ct}) = X$.

[e] Balanced differential expression as determined by microarray analysis.

study, sequences for each gene were aligned using the bl2seq tool in NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The default settings were used without the filter in order to analyze the maximum amount of sequence, and only regions with significant amounts of homology appeared in the results.

3. Results

3.1. Hybridization efficiency of bovine cDNA probes to human microarray target sequences

Probe solutions competed for hybridization to target sequences on the microarray and hybridization to a target sequence was scored successful when at least one of the probe solutions hybridized to that element. Stringency was maintained at the same level for cross-species hybridization as was used for human cDNA. In our study, bovine cDNA probes successfully hybridized to 5644 out of the 7075 non-control gene targets (79.8%), included on the microarray (data not shown), demonstrating the utility in cross-species hybridization as a means to examine bovine macrophage gene expression.

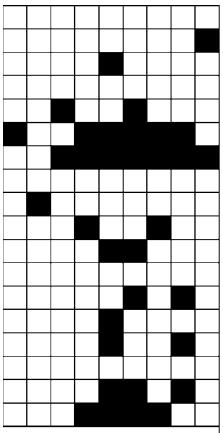
3.2. mRNA profiles in response to LPS-treatment of bovine macrophages

Balanced differential expression in monocyte-derived macrophages was determined by the ratio of LPS:control signals and balancing these values against internal control cDNAs present on the microarray. The values calculated by Incyte Genomics to define significant differential expression were $\geq +2.0$ or ≤ -2.0. Analysis of the data with respect to these cut-off values resulted in 44 (approximately 0.6%) of the target genes being differentially expressed in response to LPS treatment. Expression of 34 of these genes was increased, while 10 genes exhibited decreased expression in response to LPS treatment. Eighteen of these genes have not previously been reported to exist in cattle (Table 2).

To gain understanding of each of the gene's role in response to LPS, we analyzed each responsive gene's function using gene ontology components in the GEMtools 2.4 software package. The majority of

Table 2
Genes expressed differentially by bovine monocyte-derived macrophages in response to *E. coli* O157:H7 LPS treatment

GenBank accession number	Gene name	Symbol	BDE[a]	P1[b]	P2[c]	Macrophage[d]	Bovine[e]	Enzyme	Signal transduction	Extracellular messenger	Cytokine/chemokine	Receptor	Transcription factor	Membrane transfer	Prot. modification & maintenance	Nucleic acid synthesis	Adhesion & molecular recognition	Electron transfer	Localized & structural proteins	Metabolism	Growth & development	Apoptosis	Motility	Wound healing	Immunity & defense/inflammation
NM_002852	Pentaxin-related gene	PTX3	17.8	5858	330	YES	N																		
M10988	TNFα	TNFSF2	4.3	3608	837	YES	Y/Y																		
M31165	TNF, α-induced protein 6	TNFAIP6	4.2	707	170	YES	N																		
AF015450	CASP8 and FADD-like apoptosid regulator	CFLAR	3.9	1181	302	BM/leukocyte/spleen/tonsil	N																		
NM_001710	β-factor, properdin	BF	3.7	442	120	YES	Y/N																		
U37547	Apoptosis inhibitor 1	BIRC2	3.2	2362	740	Blood/spleen/tonsil	N																		
U37546	Apoptosis inhibitor 2	BIRC3	3.0	4228	1430	Blood/spleen/tonsil	N																		
U31511	Nitric oxide synthase 2A	NOS2A	3.0	3131	1041	YES	Y/Y																		
NM_002462	Myxovirus (influenza) resistance 1	MX1	2.9	911	316	YES	Y/N																		
M58600	Heparin cofactor II	SERPIND1	2.9	833	289	Spleen	Y/N																		
X52015	IL-1 receptor antagonist	IL1RA	2.7	2681	994	YES	Y/Y																		
U75667	Arginase, type II	ARG2	2.5	1784	724	YES	Y/N																		
D86955	Small inducible cytokine subfamily A, (Cys-Cys), member 20	SCYA20	2.5	2396	941	YES	N																		
AI906005	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor a	NFKBIA	2.4	6696	2751	YES	Y/Y																		
M17706	Colony stimulating factor 3	CSF3	2.3	9203	3930	YES	Y/N																		
NM_002164	Indoleamine-pyrrole 2,3 dioxygenase	INDO	2.3	629	271	YES	Y/N																		
AI952703	Tissue inhibitor of metalloproteinase 1	TIMP1	2.3	3069	1354	YES	Y/Y																		
M28983	Interleukin 1α	IL1A	2.3	1707	730	YES	Y/Y																		
S76638	Nuclear factor of κ light polypeptide gene enhancer in B-cells enhancer 2	NFKB2	2.2	1815	810	YES	Y/Y																		
AF026941	Homo sapiens cig 5 mRNA	cig5	2.2	589	269	Tonsil	N																		
AI191485	Inhibitor of DNA binding 2	ID2	2.2	4233	1942	YES	N																		
M24594	Interferon-induced protein 56	IFIT1	2.2	566	254	YES	N																		
AW163231	Solute carrier family 2, member 3	SLC2A3	2.2	4468	2065	BM/lung	Y/N																		
AI474055	TNF, α-induced protein 3	TNFAIP3	2.2	903	410	BM/leukocyte	N																		
F26407	Metallothionein 1L	MT1L	2.1	2939	1392	YES	Y/N																		
U25804	Caspase 4, apoptosis-related cysteine protease	CASP4	2.1	822	389	YES	N																		
BF665518	Interferon-induced transmembrane protein	IFITM1	2.1	522	252	Leukocyte/spleen	N																		
AI739106	Interferon-stimulated protein, 15 kDa	ISG15	2.1	2254	1085	Spleen	Y/N																		
M33883	Myxovirus (influenza) resistance 2	MX2	2.1	918	429	YES	Y/N																		
D28235	Prostaglandin-endoperoxide synthase 2	PTGS2	2.1	260	122	YES	Y/Y																		
M60706	Topoisomerase (DNA) I	TOP1	2.1	943	450	YES	Y/N																		
AA707391	Aminolevulinate, Δ-, synthase 1	ALAS1	2.0	258	126	YES	Y/N																		
NM_004049	BCL2-related protein A1	BCL2A1	2.0	1013	505	YES	N																		
AF083470	Interferon-induced protein with tetratricopeptide repeats 4	IFIT4	2.0	717	350	Blood/lymph	N																		
NM_000072	CD36 antigen	CD36	-3.7	572	2110	YES	Y/N																		



AA931982	Fatty acid binding protein 5	FABP5	-3.5	569	1972	Thymus/tonsil/blood	Y/N	
AB006780	Lectin, galactoside-binding, soluble, 3	LGALS3	-2.5	3165	7954	YES	Y/N	
M77349	TGF-β-induced, 68 kDa	TGFBI	-2.5	1027	2563	YES	N	
U30255	Phosphogluconate dehydrogenase	PGD	-2.4	805	1918	YES	N	
S73591	Upregulated by 1,25-dihydroxyvitamin D-3	TXNIP	-2.3	780	1760	YES	N	
NM_005606	Protease, cysteine, 1	LGMN	-2.1	2084	4438	YES	Y/N	
X56134	Vimentin	VIM	-2.0	1307	2627	YES	Y/Y	
NM_005693	Nuclear receptor subfamily 1, group H	NR1H3	-2.0	409	812	YES	N	
NM_003246	Thrombospondin 1	THBS1	-2.0	5095	9954	YES	Y/N	

[a] Balanced differential expression.
[b] P1 signal intensity.
[c] P2 signal intensity.
[d] "Yes" indicates publications listed in PubMed describing the gene in human macrophages, other terminology indicates the tissue composition of the cDNA library from which the sequence was described.
[e] "N" indicates that the gene has not been described in cattle; "Y/Y" indicates that the gene has been described in cattle, and specifically, macrophages; "Y/N" indicates that the gene has been described in cattle, but not in macrophages.
Black boxes represent molecular function, biological process, or cellular component; gray boxes represent genes whose functions have not been elucidated.

target sequences that were differentially expressed in response to LPS treatment (66%) are classified as localized and structural proteins. Other gene classifications that were highly represented in our study were metabolism, enzyme, signal transduction, growth and development, and immunity/defense/inflammation (Table 2).

We were interested in seeing if there were correlations between gene expression in response to LPS and overall levels of gene expression. To this end, we queried the data set for the 25 named target sequences with the highest LPS or control signal intensities and compared their differential expression relative to other genes in the data set. Genes that exhibited significant differential expression displayed varying signal intensities from high to low, indicating that levels of expression did not affect the differential response to LPS (Table 2), and that the concentration of RNA utilized was not supersaturating

3.3. Validated expression of selected genes by RTQ-PCR

Microarray hybridization uses fluorescence to indirectly measure the level of mRNA transcripts. Thus, we decided to independently examine the level of mRNA expression of selected transcripts by a second method to ensure the cross-species microarray hybridization results were accurate. RTQ-PCR bovine-specific primer sets (Table 1) were designed to amplify and quantitate those genes determined to be significantly altered in expression by LPS-treatment as measured by microarray analysis. The average amplicon size was 67 base pairs, and ranged from 59-98 base pairs (Table 1). Ubiquitin-C, a constitutively expressed gene, was also analyzed by RTQ-PCR and used as a normalization control for each RNA sample. The results of the RTQ-PCR assays were 90% in agreement with the results of the microarray hybridization (Table 1).

3.4. Homology between human and bovine sequences

Successful hybridization requires sufficient sequence homology between the probe solution and the target genes. Similarly, the quality of an RTQ-PCR assay is dependent upon the specificity of the primers for the sequence to be amplified. In order to

determine the similarity between the human target sequences used on the microarray and the bovine sequences used to design RTQ-PCR assays, the homology between these sequences was estimated using the NCBI bl2seq tool. The sequence homology between the two species for all but two genes analyzed ranged from 76 to 96%, with a mean homology of 86% (Table 1).

4. Discussion

The purpose of this study was to use a human gene microarray to identify bovine macrophage genes that differentially respond to *E. coli* O157:H7 LPS. Lack of widely available, high quality, bovine gene microarrays is a major obstacle in examining global gene expression responses in bovine cells. It was our hypothesis that utilizing human gene microarrays would provide a means to examine bovine cell gene expression. The stimulation of macrophage gene expression by LPS is a well-characterized system that would allow us to evaluate the validity of the cross-species hybridization technique. In addition, gene expression resulting from stimulation with LPS has been shown to overlap with that resulting from stimulation with whole bacteria [17], and would, thus, provide insight into the specific responses of bovine cells to *E. coli* O157:H7, an important pathogen of humans.

Macrophages are active cells that elaborate a large number of cytokines and growth factors required for antigen processing and presentation, as well as cell survival. Bacterial LPS triggers the increased expression of a number of inflammatory cytokines and factors involved in the bactericidal activity of macrophages [18,19]. Previously identified constitutively expressed macrophage genes with roles in coagulation, complement activation, plasma and matrix function, cell motility, and growth successfully hybridized with the bovine cDNA [20]. Of the 44 differentially expressed bovine genes identified in this study, all have been previously identified in human macrophages or human macrophage-rich tissues (Table 2). We found the PTX3, MX1, NFKB2, MT1L, and NFKB1A genes elevated in response to LPS treatment. All of these genes are known to have a role in macrophage activation or bactericidal activity in response to LPS in humans or mice [21–26]. In addition, the macrophage genes TNFα and nitric oxide synthase 2 (NOS2) exhibited the responses to LPS described in earlier reports [27–30]. Together, these data demonstrated the utility of using human gene microarrays for the analysis of bovine macrophage gene expression.

We identified 44 LPS-responsive genes in this study. It is possible that varying the conditions of treatment may vary the number of responsive genes and the level at which they respond. In this study, monocyte-derived macrophages were treated with 10 μg/ml of *E. coli* O157:H7 LPS for 1 h. It is entirely possible that a different list of genes may have been compiled if the cells were incubated with a different *E. coli* strain of LPS, for a different period of time with the same LPS, or with a different LPS concentration. Additionally, a number of genes that were quiescent in mature macrophages and up-regulated upon stimulation with LPS may have been masked due to the artificial stimulation during adherence of the macrophages to the flasks [31–33]. Finally, some messages may be highly unstable and have degraded rapidly prior to isolation. For instance, in studies using RAP-PCR, different results were obtained when epithelial cell cultures were treated with cyclohexamide to stabilize rapidly labile mRNA, compared to cultures where cyclohexamide was not used [1].

Variation in results may also be attributable to differences in age, health status, and/or genetics of the cattle population selected for macrophage isolation. Within our study, we controlled for these factors by pooling RNA from 44 individuals to minimize the effect of "outlying" animals. In addition, we performed a preliminary microarray experiment using a pool of RNA from monocyte-derived macrophages obtained from 35 different cattle and found expression profiles similar to those described in this experiment (data not shown). Thus, we feel confidant that we have identified LPS responsive genes.

As an additional verification that we were specifically measuring differential expression due to LPS treatment, we compared our data to that of a colleague studying bovine cell gene expression in a non-macrophage, non-LPS-treated system using human microarrays from Incyte Genomics. The 44 genes differentially expressed in our system were not

differentially expressed in their studies (Dr Tim Smith, personal communication). Thus, the results of our studies cannot be attributed to artifactual hybridization of bovine cDNA to the human genes on the array.

Rajeevan et al. have reported that real-time PCR validated 71% of the genes identified by a human microarray, suggesting that expression results from these experiments must be further validated [34]. In our experiment, RTQ-PCR assays using bovine-specific primers were in greater than 90% agreement with the results obtained using a human microarray. This not only provides further validation that the hybridization of bovine cDNA to human gene microarray targets is indeed specific, but also that under the conditions used in our study, was as reliable as an experiment carried out using cDNA from a homologous species.

In a study designed to estimate the cross-hybridization of similar genes on microarrays, Evertsz et al. determined that targets containing greater than 80% identity to the hybridization probe sequences showed cross-reactivities ranging from 26 to 57%, with percent sequence identity being the best predictor of hybridization cross-reactivity [15]. They predicted that sufficient homology exists between species for human microarrays to be used in cross-species experiments [15]. Out of the 42 genes for which we determined the homology between the human and bovine sequences, only two, SCYA20 and ISG15, had identities less than 80% (Table 1). The bovine sequences chosen to design RTQ-PCR primers for CD36 and ubiquitin C were selected by searching for bovine sequences specific for those genes, and not due to homology with the target sequence on the microarray. Although these genes share homology in their entirety, these particular sequences do not. It is notable that although the results of three of the RTQ-PCR assays did not agree with those of the microarray (BF, ISIG15, and CD36), the sequence homology for these genes ranged from 76 to 89% (Table 1). However, because the human gene cDNAs used as targets on the microarray ranged in size from 500 to 5000 base pairs, our estimates of homology between the bovine and human sequences may in reality be greater or less than what actually existed in these experiments.

This is the first report of bovine-human cross-species expression profiling by microarray hybridization. We have identified 44 genes that differentially respond to *E. coli* O157:H7 LPS in bovine macrophages. Of these 44 genes, 18 have not previously been reported in cattle and an additional 17 have not previously been studied in bovine macrophages. The pentaxin-related gene has been identified in bovine cells for the first time and responds dramatically to LPS treatment. These results demonstrate that the use of microarrays manufactured with human target sequences facilitate the analysis of bovine gene expression. Until such time as bovine species-specific microarrays become widely available, human microarrays may provide a viable alternative to traditional assays for screening large numbers of genes for changes in expression or identification of characterized human genes as yet unidentified in the bovine genome.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Stacy Bierman, Tammy Sorensen, and Ron Mlejnek. We thank Joan Rosch and Sherry Kluver for secretarial assistance, the MARC cattle operations staff for assistance with sample collection, and Drs Tim Smith, Mike Clawson (MARC), and Frank Blecha (Kansas State University, Manhattan, KS) for critical review of the manuscript. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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